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Article

Fe(III) - Galactomannan Solid and Aqueous Complexes. Potentiometric, EPR Spectroscopy and Thermal Data

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Galactomananas podem ser empregadas como aditivos na indústria alimentícea para modificar a viscosidade do produto final. Como não são absorvidas pelo organismo humano, sua adição a alimentos dietéticos é muito promissora. Os equilíbrios entre Fe(III) e galactomananas e arabinogalactana de várias leguminosas foram caracterizadas por titulações potenciométricas e espectroscopia de EPR. Os logaritmos das constantes de equilíbrio para a formação dos complexos ML (M = Fe(III) e L = unidade monomérica dos biopolímeros) foram iguais a 15,4, 14,1 e 18,5 para as galactomananas de *C. fastuosa, L. leucocephala e S. macranthera*, respectivamente. Os valores de log *K* para a formação das espécies protonadas (MHL) foram 3,1, 3,3 para as galactomananas de *C. fastuosa, L. leucocephala* e não foi detectado no caso da galactomanana de *S. macranthera.* Os valores de log de *K* para a formação de ML_2 foram 14,1, 13,3 e 15,2, respectivamente. Não foi possível obter dados relativos ao equilíbrio entre arabinogalactana e Fe(III) devido à formação de produtos insolúveis logo no início das titulações. Os ensaios no estado sólido mostraram não somente uma grande interação dipolar entre dois íons Fe(III) na estrutura polimérica complexada, tanto maior quanto menos substituída é a galactomanana, mas também a estabilidade térmica resultante desta complexação. Os produtos complexados podem conferir uma abrangência maior na utilização em produtos dietéticos, pois a presença de um metal essencial no complexo, quando de sua descomplexação nos vários processos fisiológicos dos organismos, faculta a absorção desse íon metálico.

Galactomannans can be employed in food industries to modify the final rheological properties of the products. Since they are not absorbed by the living organisms they can also be used in dietary foods. The equilibria involving the interactions of Fe(III) and galactomannans and arabinogalactan of several leguminous plants were characterized by potentiometric titrations and EPR spectroscopy. The log of the equilibrium constants for the formation of ML species, where M is the metal ion and L is the monomeric unit of the biopolymers, were 15.4, 14.1 and 18.5, for the galactomannans of *C. fastuosa, L. leucocephala* and *S. macranthera*, respectively. Log *K* values for protonated species (MHL) were 3.1, 3.3, and were not detected for the galactomannan of *S. macranthera.* The log *K* values for the formation of ML_2 were 14.1, 13.3 and 15.2, respectively. Early formation of insoluble products in the equilibrium with arabinogalactan and Fe(III) prevented acquisition of reliable data. The solid complexes assays showed a great dipolar interaction between two Fe(III) ions in the inner structure of the biopolymer which increased as the degree of substitution of the galactomannan decreased, and also showed the resulting thermal stability. The complexes impart a new possibility of providing essential metal ions in dietary foods since decomplexation of the complexes can occur at different pH values existing in the human body.

Keywords: iron (III), potentiometric titration, binding constants, EPR spectroscopy, metal ion coordination

Introduction

Galactomannans are present in the endosperm of seeds of Leguminosae family. The ratio between the galactose and mannose monomers varies according to the species and is also affected by climate, thus providing different substitution degrees (SD) in the biopolymer. Galactomannans are used in many industrial processes as an alternative to guar gum, locust beam gum and other gums, taking advantage of the fact that their rheological properties and their solubility are dependent upon their SD[1-7].

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In Figure 1 a scheme of a galactomannan of mannose to galactose average ratio of 1:3 is shown. Arabinogalactan is an edible polysaccharide[8-11].

Iron is involved in many complex biological processes, not only in oxidation-reduction catalysis and bioenergetics, but also in many acid-base reactions[12-15] and when complexed to biopolymers it affects their properties described above.

The ability of galactomannans and arabinogalactans to chelate metal ions is of interest, since their use in food industry could lead to the provision of an essential metal ion in dietary foods[16].

The discovery by Angyal[17] that the conformations of sugars in the solid state differ from those in solution prompted many studies of equilibria between sugars and metal ions in the solid state[5-7,10-11,18-23]. Solid complexes of Fe(III) with various monosaccharides, including galactose and mannose, have been reported to have varying stoichiometries[24]. In solution these complexes are mononuclear (1:1) or dinuclear (1:2) at pH above 13, whereas polymeric complexes exist at low pH values.

The present work aimed to determine the binding constants and speciation of Fe(III) with galactomannans of *Cassia fastuosa, Leucaena leucocephala* and *Senna macranthera* and arabinogalactan from *Pereskia aculeata*, and to monitor the extension and ability of forming complex species as a function of pH. Potentiometric titrations were used in aqueous solutions, whereas EPR spectroscopy and thermal analyses were used to study the complexes in the solid state. The data will help to understand the regulation of the Fe(III) absorption in dietary foods based on the ability of forming the complexes, as variations in pH values in the physiological processes of a human body occur.

Experimental

All chemicals were of analytical-reagent grade and were used without further purification. CO_2^- free distilled and deionized water was used in all solutions.

The galactomannans were obtained by aqueous extraction of seeds from *C. fastuosa, L. leucocephala* and *S. macranthera*, and arabinogalactan by aqueous extraction of the leaves of *P. aculeata*[5-6,25]. Alditol acetate derivatives of both polysaccharides were prepared and analyzed by GLC-MS (Varian 3300) with an OV-225 capillary column (0.25 mm id x 30 m) linked to a Finnigan Trap (model 419) mass spectrometer at 70 eV. Injection was done at 50 °C, followed by a programmed rise of 4.0 °C min^{-1} to 220 °C[26-27].

Stock solutions of 5g of polysaccharide per liter were prepared for all four polysaccharides. Aliquots containing the required number of millimoles of monomer were transferred to the reaction mixture. The number of millimoles was calculated by averaging the molecular masses of the monomers, using the proportions of the two monomers to weight the average. The aqueous metal ion solution was prepared by addition of Fe(III) (referred throughout this work as M) nitrate hexahydrate ($Fe(NO_3)_3.6H_2O$ - Merck - Brazil) to a 0.3 mol L^{-1} aqueous solution of HNO_3. The content of Fe(III) was determined by titration with EDTA, using variamine blue as indicator[28], and the H^+ content was determined by Gran's Plot[29].

The aqueous solution of the titrant, KOH, was prepared from pellets from a fresh bottle (Merck - Brazil) and standardized with potassium acid phthalate (Carlo Erba - Brazil). KNO_3 (Baker & Adamson - USA) was used as the supporting electrolyte to maintain the ionic strength(I) at 0.100 mol L^{-1}.

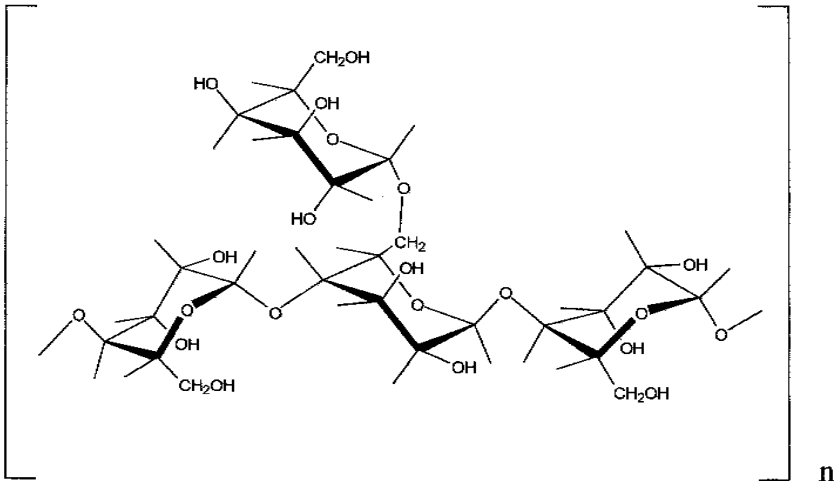

Figure 1. Proposed structure of a galactomannan of average 3:1 mannose to galactose ratio.

All potentiometric titrations were performed in triplicate under a stream of purified nitrogen and carried out with an Orion model 420-A research grade pHmeter (USA) fitted with a glass, H^+ sensitive, electrode, and a Ag/AgCl reference electrode (Orion - Switzerland) calibrated with standard HCl 0.01 mol L^{-1} [I=0.100 mol L^{-1} (KNO_3)] to read the -log of the concentration of H^+ directly, following the procedures described in the literature[29]. The temperature was maintained at (25.0 ± 0.1)ºC (MQBTC 99-20, Microquímica - Brazil).

Volumes of (0.02 ± 0.01) mL of the titrant were delivered by a Sigma® Techware® Digitrate (USA) manual piston burette.

The solid complexes were obtained by addition of a KOH solution to warm aqueous solutions of the biopolymers 10 times the number of millimoles of the metal ion added so as to reach a pH around 9.0. After cooling, the solutions were centrifuged and the solids dried in an oven at 60ºC.

The Electron Paramagnetic Resonance (EPR) spectra of powdered solid native and complexed polysaccharides were registered at room temperature (25ºC) in quartz tubes. A Bruker ESP 300 E spectrometer (Germany) was used operating at a frequency of 9.7 GHz (X-band), with a 100 KHz modulation frequency, 2.024 G modulation amplitude and ~20 mW microwave power. Simulations of EPR spectra were carried out using Win-EPR® and SimFonia programs®.

The Thermogravimetry - Differential Scanning Calorimetry (TG - DSC) analyses were recorded in a Netzsch simultaneous Thermal Analysis STA 409 EP (Germany), under air, from 21ºC to 520ºC, at 2ºC min^{-1}, using open cylindrical aluminum sample pans, 4 mm diameter, 2 mm high.

All equilibrium constants were calculated using the microcomputer program Best7[29]. For the refinement of the binding constants, BEST7 uses as input the hydrolysis constants of the metal ions employed[30], the protonation constants for the binding sites of the ligand, the pK_w under the experimental conditions and the number of millimoles of all reagents at equilibrium.

The basic algorithm in Best7[29] is:

$$T_i = \sum_{j=1}^{NS} \left(e_{ij} \beta_j \prod_{k=1}^{i} [C_k]^{eij} \right) \quad (1)$$

which is a statement of the mass balance of the i-th component in terms of the j-th species summed over all species present, (NS). Each species concentration consists of a product of the overall stability constant and individual component concentrations $[C_k]$ raised to the power of the stoichiometric coefficient e_{ij}. The set of simultaneous equations obtained is solved for each component $[C_k]$. The value of $[C_k]$ represents the calculated concentration of H^+, which is then compared with the measured hydrogen ion concentration. The standard deviation in pH units is obtained from equation 2:

$$\delta_{fit} = (U/N)^{½} \quad (2)$$

where $N = \Sigma w$ and equations 3 and 4:

$$U = (pH_{obs} - pH_{calcd})^2 \quad (3)$$

$$w = 1/(pH_{i+1} - pH_{i-1})^2 \quad (4)$$

The mathematical algorithm in Best7 sets equations which are solved by minimizing δ_{fit} in order to provide the overall stability constants[29] for the system studied.

The species distribution diagrams were drawn using the output data of Best7 by another microcomputer program, SPE[29] .

Results and Discussion

Silica gel thin layer chromatography of the extracts, in combination with monomeric sugar standards, showed that the extracted biopolymers were free from major impurities. The average mannose to galactose ratios, obtained by GLC-MS of the alditol acetate derivatives, were 3.7:1, 2.6: 1 and 4:1 for the galactomannans extracted from *Cassia fastuosa*, *Leucaena leucocephala* and *Senna macranthera*, respectively. Galacturonic acid substitutions were present in the galactomannan of L. leucocephala. For the arabinogalactan of *Pereskia aculeata* the arabinose to galactose ratio was 1:1.4.

The mathematical model that best described the chemical aspects of the equilibria of metal-polysaccharide complexes comprises two *cis*-hydroxyl groups that are depleted of their protons on each of the monomeric sugar binding units[14,15,18,19]. In this manner each monomeric unit provides two basic sites to complex the metal ion. This complexation of metals by saccharides through deprotonated hydroxyl groups is confirmed by the spectroscopic properties of mono- and disaccharide complexes of Fe(III)[34].

The first protonation constant for the monomeric unit of mannose is too high to be determined by potentiometric titrations. Therefore a literature value, determined by UV-Vis spectroscopy[35] and related to the hydroxyl group in the C-6 was used . The second protonation constant was calculated using the program Best7 and was attributed to C-3, a *cis* hydroxyl group to C-6 . The equilibria for these protonation constants are:

$$^-O\text{-}L\text{-}O^- + H^+ \rightleftharpoons HO\text{-}L\text{-}O^- \quad \log K_{a1} = 12.60 \pm 0.2^{35} \quad (5)$$

$$HO\text{-}L\text{-}O^- + H^+ \rightleftharpoons HO\text{-}L\text{-}OH \quad \log K_{a2} = 10.60 \pm 0.2 \quad (6)$$

where $^-O\text{-}L\text{-}O^-$ is the deprotonated sugar unit, hereafter referred to as L, H^+ is a proton, hereafter represented without the charge, as H, $HO\text{-}L\text{-}O^-$ is the singly protonated sugar unit, hereafter referred to as HL, and HO-L-OH is the doubly protonated sugar unit, hereafter referred to as H_2L.

Potentiometric titrations

Potentiometric titrations were not successful in determining the constants equilibria of galactose and mannose monomers and Fe(III) in this work.

The potentiometric titration profiles, some of which are presented in Figures 2 and 3, were used to determine the binding constants listed in Table 1. The profiles for the galactomannan from *C. fastuosa*, in the presence of 0.1 and 0.05 mmol of Fe(III), were different from those typically seen from the Fe(III)-ligand systems, in which insoluble iron hydroxides or insoluble iron-ligand complexes normally form around pH 5-6. In the present work such insoluble products were not observed during the titrations. Titration of the galactomannan from *S. macranthera* in the presence of Fe(III) also showed this behaviour (data not shown) with the buffer region occurring at pH around 8. In the potentiometric profiles the base necessary to neutralize the strong acid added to the metal ion solution to take them to pH values below 1.80 is not included in the calculation of the X-axis values.

Figure 3 presents the titration profiles of the most substituted of the three galactomannans, that of *l. leucocephala*. The break is the profile in the absence of Fe(III) is less steep than those obtained in the corresponding titrations with the other two galactomannans. This feature along with the lower initial pH value for the titration was probably due to the presence of galacturonic acid in the structure. In the presence of Fe(III), insoluble products were formed at lower pH values than for the other two galactomannans.

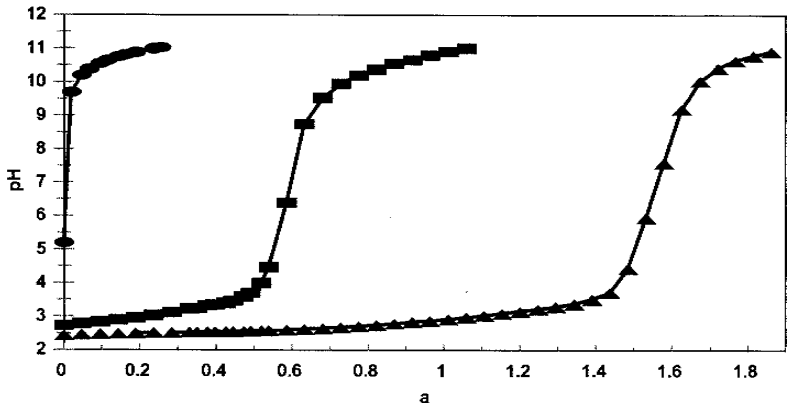

Figure 2. Potentiometric pH profile of a solution of 1 g L^{-1} galactomannan from *C. fastuosa* (●) with 0.05 (■) and 0.10 mmol (▲) of Fe(III). T = 25.0ºC and I = 0.100 mol L^{-1} (KNO_3). a = ratio between the number of mmol of KOH and the number of mmol of ligand.

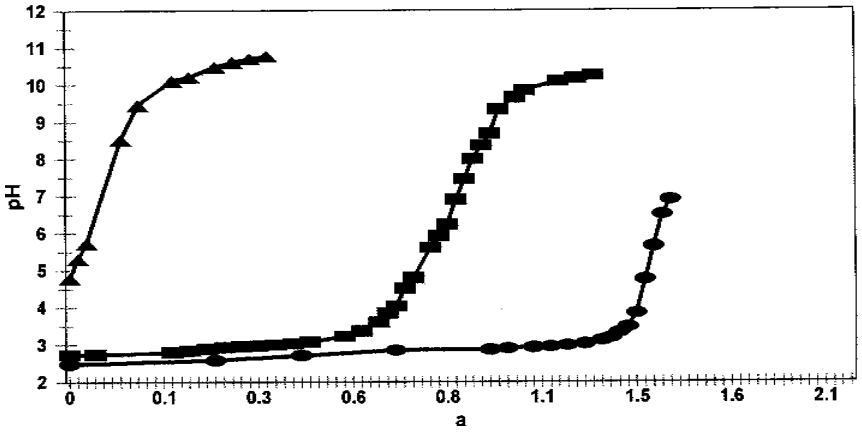

Figure 3. Potentiometric pH profile of a solution of 1g L^{-1} galactomannan from *L. leucocephala* (▲) with 0.05 (■) and 0.10 (●) mmol of Fe(III). T = 25.0ºC and I = 0.100 mol L^{-1} (KNO_3). a = ratio between the number of mmol of KOH and the number of mmol of ligand.

Table 1. Logarithms for the binding constants, K_{app}, of the complexes of galactomannans and Fe(III). T = 25.0ºC and I=0.100 mol L^{-1} (KNO_3).

galactomannans from: log K_{app}	*C. fastuosa*	*L. leucocephala*	*S. macranthera*
man:gal ratio (average degree of substitution)	3.7:1	2.6:1	4:1
[ML]/[M][L]	15.4± 0.5	14.1± 0.5	18.5± 0.5
[MHL]/[ML][H]	3.1± 0.5	3.3± 0.5	n.d.
$[ML_2]$/[ML][L]	14.1± 0.5	13.3± 0.5	15.2± 0.5

n.d.: not detected

Table 1 shows that the metal-polymer complexes are remarkably stable. The least substituted galactomannan, that of *S. macranthera*, had the highest binding constant for the equilibrium M + L $\rightleftharpoons$ ML. The binding constant values for this equilibrium decreased as the degree of substitution of the galactomannan increased. A similar pattern occurred with binding constant values for the equilibrium ML + L $\rightleftharpoons$ ML_2. When the arabinogalactan from *P. aculeata* was titrated in the presence of Fe(III), insoluble products formed at pH values below 3.0, making it impossible to calculate the binding constants. Protonated ML species (ML + H $\rightleftharpoons$ MHL) were found for galactomannans of *C. fastuosa* and *L. leucocephala* with similar values for the logarithms of the binding constants.

The species distribution curves for the galactomannan from *C. fastuosa* with the Fe(III) concentration ratio of 1:2 (M:L), total metal concentration set at 100%, showed that the protonated form of the ML (1:1) species (*i.e.* MHL) reached a maximum at pH=2.0, where it accounted for 92.2% of the iron complexed. The unprotonated form, ML, reached a maximum at pH=3.1, representing 27.2% of the iron complexed. Finally the species ML_2 reached its maximum at pH=10.1, representing 84.8% of the iron complexed. Similar species distribution curves were obtained upon titrations of the *L. leucocephala* galactomannan in the presence of Fe(III). The curves obtained for the *S. macranthera* galactomannan, with an Fe(III) concentration ratio of 1:2 (M:L), total metal concentration set at 100%, showed ML reaching a maximum at pH=2.0 where it represented 66.4% of the iron complexed and ML_2 reaching a maximum at pH=8.3, where it represented 56.6% of the iron complexed. All the titrations in the presence of Fe(III) started at pH near 1.8, where the free metal ion concentration is above 40%, although the distribution diagrams show a small percentage of Fe(III) at pH above 2.0.

Thermal analysis

Thermal analysis by TG-DSC of the solid native galactomannan and the solid complex formed between Fe(III) and the *C. fastuosa* galactomannan did not show any remarkable endothermic transition between room temperature and 225 ºC, indicating the absence of any crystalline or other phase change during heating. A peak centered at 250 ºC for the complex and at 290 ºC for the native galactomannan indicates an exothermic process possibly linked to a change in conformation and the breakage of some of the polymer. A final destructive oxidative process occurs at 425 ºC for the native polymer and at 470 ºC for the complexed polymer (numbered 3 in Figures 4 and 5). Of the three galactomannans studied, this was the only one for which the final oxidation occurred at a lower temperature for the native biopolymer than for the complexed polymer.

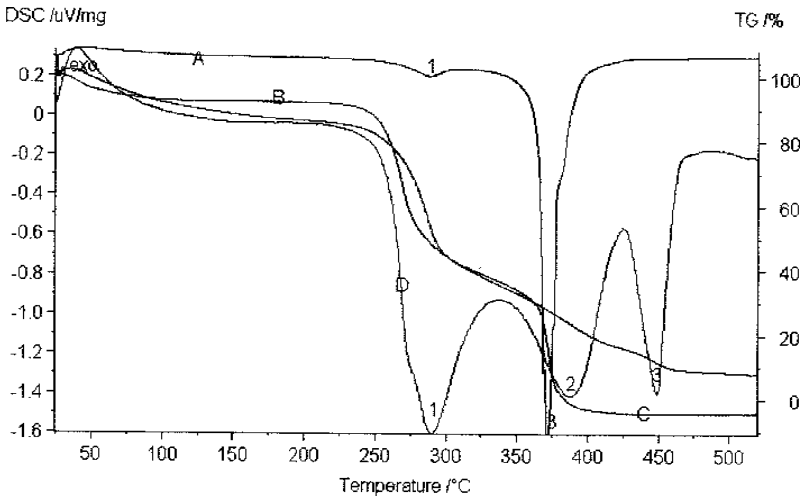

Figure 4. Thermal profiles of the solid products extracted from an aqueous solution of pH 8.0 - 9.0, 10 moles galactomannan from *L. leucocephala* to 1 mole of Fe(III). A and C are the DSC and TG profiles of the complexes and B and D, the TG and DSC profiles of the galactomannan, respectively. See text for 1, 2 and 3.

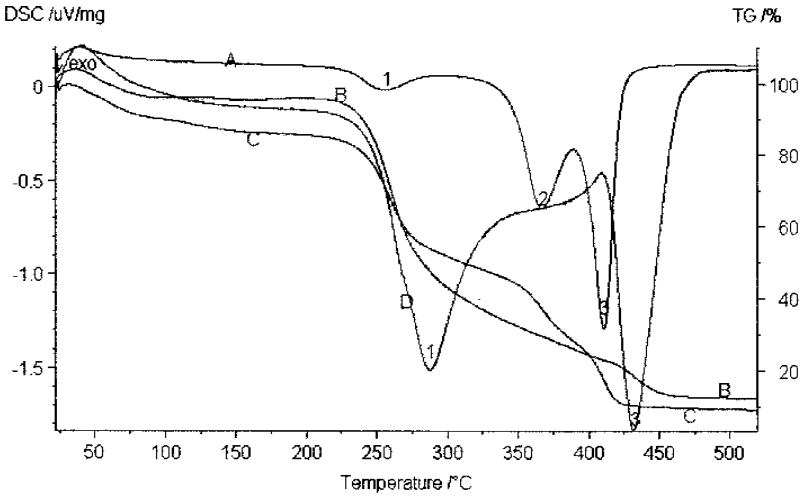

Figure 5. Thermal profiles of the solid products extracted from an aqueous solution of pH 8.0 - 9.0, 10 moles galactomannan from *S. macranthera* to 1 mole of Fe(III). A and C are the DSC and TG profiles of the complexes and B and D, the TG and DSC profiles of the galactomannan, respectively. See text for 1, 2 and 3.

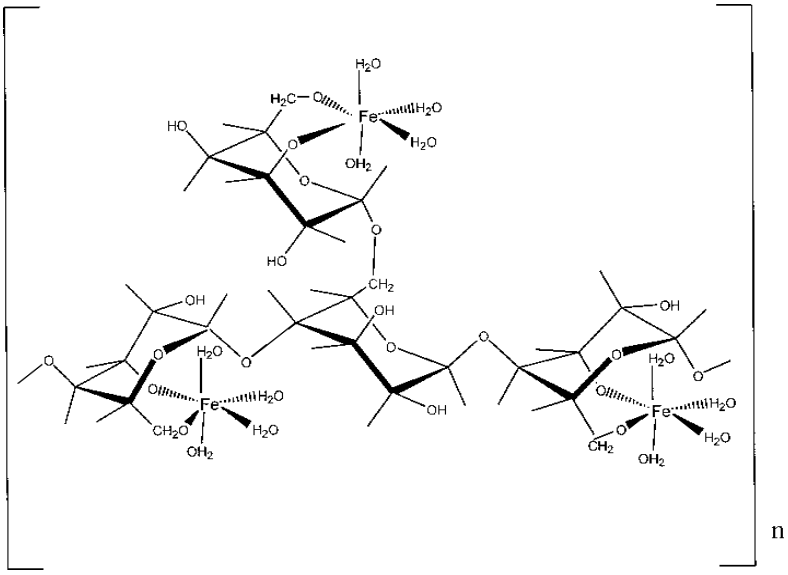

Figure 6. Proposed structure for the complex of a galactomannan average 3:1 mannose to galactose ratio and Fe(III).

The native *L. leucocephala* galactomannan did not show any remarkable thermal effect until 290 °C, where there was a break of chains, associated with a large loss of mass (Figure 4 - peak number 1). There was also a conformation change near 390 °C, but without any significant loss of mass (Figure 4 - peak number 2). The final oxidative process occurred at 445 °C (Figure 4 - peak number 3). The final oxidative exothermic peak for the Fe(III)-galactomannan complexes occurred at 370 °C. None of the conformational changes of the native biopolymer occurred with the complex, which showed only a very small DSC peak, with the TG curve showing a great loss of mass (290 °C). Although the binding constant for the ML complex in solution for this galactomannan was the lowest of the three studied, in the solid state this complex had the strongest interaction.

The native *S. macranthera* galactomannan presented a conformational change and break of chains at 280°C, while this process occurred at 255 °C for the galactomannan-Fe(III) complex (Figure 5 - peak number 1). The complex showed another conformational change in the 365-385 °C, temperature range with no significant loss of mass (Figure 5 - peak number 2). The final oxidation occurred at 410 °C for the complexed galactomannan and 430 °C for the native galactomannan (Figure 5 - peak number 3) . This galactomannan has a more mobile complexed chain than that of *L. leucocephala* (more thermal events than the latter), even though it has a higher binding constant.

The native arabinogalactan from *P. aculeata* showed an endothermic peak centered at 150 °C, due to a crystalline or other phase change. In the case of the complexed

arabinogalactan this process occurred at 165 ºC. In both cases there was a conformational change and breakage of chains, that occurred in the temperature range of 270-350ºC for the complex and 295-390ºC for the native arabinogalactan. The final oxidative process occurred at 410ºC and 430ºC for the complex and the native arabinogalactan, respectively.

The exothermic peaks at the beginning of all runs are due to the loss of absorbed water.

EPR spectra

The EPR spectrum of the galactomannan from *L. leucocephala* presented traces of rhombic (isolated high-spin Fe(III)) (g = 4.30) and octahedral (hexacoordinated) Fe(III) (g = 2.05) arising from strong interactions between the paramagnetic, high spin Fe(III) centres complexed to sugar-type ligands[34], as well as some free radicals (g=2.00) arising from organic impurities in the extracted galactomannan. The spectra of the galactomannans from *C. fastuosa*[5] and *S. macranthera* also presented some octahedral (hexacoordinated) Fe(III) sugar-type complexes and some hexacoordinated Fe(III) complexed to impurities (g = 2.17). Table 2 presents all the EPR parameters obtained.

Table 2. EPR spectra parameters of the complexes of galactomannans and arabinogalactan with Fe(III).

EPR param.	g	A(G)	$g_{\parallel}$	$A_{\perp}$ (G)	$g_{\perp}$	ΔHp_p	$g_{free\ radical}$
C.	2.004	-	-	-	-	1060	2.003
L g	-	-	2.26	30	2.05	-	2.005
L	2.074	-	-	-		780	2.002
S	2.39	-	-	-		1300	-
P a	2.0	100	-	-		-	

*C = C. fastuosa - Fe(III) com*plexes; L g = galactomannan of *L. leucocephala;* L = *L. leucocephala* - Fe(III) complexes; S = *S. macranthera* - Fe(III) complexes; P a = arabinogalactan of *P. aculeata*

The ability of Fe(III) to complex deprotonated hydroxyl groups of saccharides is well known[34]. The ΔH_{pp} (line width) values (Table 2), which monitor the strength of the dipolar interaction of Fe(III) ions inside the web structure of the biopolymer, indicate that the average distance between Fe(III) ions is larger for the more substituted galactomannans. Combining these results with the TG-DSC results, it appears that a short distance between the metal ions within the complexed biopolymer leads to a greater mobility of the chains.

The EPR spectrum of the Fe(III) complexes of arabinogalactan from *P. aculeata* presents rhombic and octahedral Fe(III) signals as in the case of the galactomannans, and some signals probably due to proteinic material. This proteinic contaminant has been described in the literature[36-37] as a common impurity when extracting polysaccharides, and in the case of arabinogalactan has rendered it impossible to distinguish the EPR parameters in the complexes.

Conclusions

The species distribution curves of the galactomannans and Fe^{3+} showed the existence of complexed species from very acid pH values to very basic ones (2.0 to 10.0). This ability to form complexes over a large range of pH values makes it possible to use this material within this pH range. There may be complexed species in aqueous solution other than those found in this work, but they represent less than 10% of the total of all species present. Below this percentage the results are not considered reliable by the methodology employed[29].

In the TG-DSC, the complexed galactomannans tended to have fewer thermal events than the native galactomannans, although more thermal events became apparent for the galactomannans with lower degrees of substitution. The difference between the final degradation temperatures of the native galactomannan and the complex also increased with the decrease in degree of substitution. Although the complex of the most substituted galactomannan had the lowest ML binding constant in aqueous solution, it also had the greatest resistance to thermally induced conformation changes. On the contrary, in relation to the native polymer, the complexation of Fe(III) with arabinogalactan let to a decrease in the temperature at which the thermal events occurred. Structural changes induced by chelation processes may have one of two opposing effects on the thermal behavior of the polymer[38]: the metal ion may disturb the natural conformation, leading to thermal unstability, or it may provide a bridging effect, thereby enhancing thermal stability. The current work has shown both these effects.

EPR spectroscopy showed that the least substituted galactomannan, that of *S. macranthera* hold more Fe(III) ions in its web like structure. The higher degree of complexation with the higher ratio of mannose to galactose (average degree of substitution) suggests that mannose is the sugar unit that is mainly involved in the formation of the complexes, in both the aqueous and solid phases.

Although biopolymers are not uniform compounds, the viability of using potentiometric titrations in the determination of binding constants of complexes between metal ions and biopolymers has recently been demonstrated[5-7,11,20-22].

Galactomannans impart rheological modifications in the product to which they are added. These studies presented new perspectives to the use of biopolymers

complexed to an essential metal ion in food industries over a wide range of temperatures in industrial processes, since as long as changing the viscosity it can provide the essential metal ion Fe(III) in dietary edible products as it can be released from the complexes at the various pH values of the human body physiology.

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